DOCKET NO.: 217301US0PCT
SERIAL NO.: 10/009,782

MARKED-UP COPY OF AMENDMENT

IN THE SPECIFICATION

Please substitute the attached substitute specification for the original specification.

~~Description~~

~~Title of the Invention~~

TITLE

TRANSFORMED MICROORGANISM AND PROCESS FOR PRODUCING D-AMINOACYLASE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a national-stage filing under 35 U.S.C. §371 of PCT/JP00/03932, filed June 15, 2000. This application claims priority under 35 U.S.C. §119 to JAPAN 11/17055, filed June 17, 1999.

REFERENCE TO SEQUENCE LISTING

This application contains a sequence listing of nucleic acid and amino acid sequences.

~~Technical Field~~

BACKGROUND OF THE INVENTION

Field of the invention

The present invention relates to a transformed microorganism prepared by inserting into a zinc-tolerant microorganism a D-aminoacylase-producing gene which selectively produces D-aminoacylase alone between D-aminoacylase and L-aminoacylase, and a process for producing D-aminoacylase by utilizing the transformed microorganism.

~~Background Art~~

Description of the Related Art

D-aminoacylase is an industrially useful enzyme ~~industrially useful~~ for the production of D-amino acids of high optical purity, which are ~~needed for uses in side chains of~~ used for the side chains of antibiotics, peptide drugs and the like.

Chemical and Pharmaceutical ~~Bulletinn~~ Bulletin 26, 2698 (1978) discloses Pseudomonas sp. AAA6029 strain as a microorganism simultaneously producing both D-aminoacylase and L-aminoacylase.

Japanese Patent Application Laid-open No. Sho-53-59092 discloses actinomycetes, such as Streptomyces olibaceus S•6245. The use of these microorganisms results in the simultaneous production of both ~~the~~ optical isomers of aminoacylase, D-aminoacylase and L-aminoacylase~~, apart from the potency to produce D-aminoacylase~~. While these organisms are capable of producing D-aminoacylase, it is necessary to separate this enzyme from its optical isomer, L-aminoacylase. Thus, laborious and costly procedures are disadvantageously required for the separation of the two.

~~Alternatively, for example,~~ Japanese Patent Application Laid-open No. Hei-1-5488 discloses Alcaligenes denitrificans subsp. xylosoxydans M1-4 strain as a microorganism that selectively ~~producing~~ produces D-aminoacylase alone. ~~In case~~

~~that~~ If this bacterial strain is utilized, no laborious work is required for the separation of D-aminoacylase from L-aminoacylase. However, the ~~potency~~ capacity of ~~the~~ this bacterial strain to produce D-aminoacylase is insufficient. Furthermore, the nucleotide sequence of the D-aminoacylase-producing gene is not elucidated in Japanese Patent Application Laid-open No. Hei-1-5488. Thus, this document does not describe how to modify the D-aminoacylase ~~no modification of the~~ gene so as to improve ~~the~~ its D-aminoacylase-producing ~~potency~~ capacity or ~~no~~ describe the creation of a transformed bacterium with ~~a high productivity has been accomplished~~ an ability to produce higher amounts of D-aminoacylase.

BRIEF SUMMARY OF THE INVENTION

~~Under such circumstances, the~~ In view of the above, the present inventors Moriguchi, et al. elucidated the structure of the D-aminoacylase-producing gene in the Alcaligenes xylosoxydans subsp. xylosoxydans A-6 strain and demonstrated its nucleotide sequence, which appears as ~~of~~ SEQ ID NO: 1 in the sequence listing. Further, ~~a certain~~ it was found that genetic modification of the D-aminoacylase-producing gene successfully improved the D-aminoacylase-producing ~~potency~~ capacity of the resulting transformed bacterium (Protein Expression and Purification 7, 395-399 (1996)).

Brief Description of the Drawings

Fig. 1 schematically depicts the plasmid used for ligation with the D-aminoacylase-producing gene.

Fig. 2 schematically depicts the plasmid ligated with the D-aminoacylase-producing gene.

~~Disclosure of the Invention~~

DETAILED DESCRIPTION OF THE INVENTION

The inventors' subsequent research works have elucidated that the D-aminoacylase-producing ~~potencies~~ capacities of various transformed bacteria with the aforementioned D-aminoacylase-producing gene inserted therein are greatly improved in zinc ion-containing culture media. It has also been found that the D-aminoacylase producing ~~potencies~~ capacity of a transformed bacterium is ~~are~~ prominently improved by controlling the zinc ion concentration within a predetermined range~~, in particular~~.

Furthermore, it has been found that the above-mentioned effect varies significantly depending on the type of a host microorganism and that a host microorganism with high such effect generally exerts zinc tolerance even prior to the transformation thereof. Herein, the term "zinc tolerance" means that the growth potency of a bacterium as measured on the basis of the cell weight (A660 nm) is hardly inhibited by the addition of

zinc ion.

The findings mentioned above indicate the following two points: ~~followings (1) and (2).~~ (1) The expression of a transformed microorganism with a D-aminoacylase-producing gene of SEQ ID NO: 1 ~~in the sequence listing~~ is enhanced in the presence of a given quantity of zinc ion, though the reason has not been elucidated. (2) Since it is believed that zinc ion functions in an inhibiting manner on common microorganisms, a congenitally zinc tolerant microorganism should be selected as a host to insert the gene therein so as to sufficiently procure the effect of zinc ion.

Based on the above-mentioned points, the invention provides a microorganism transformed with a D-aminoacylase-producing gene, the D-aminoacylase-producing ~~potency~~ capacity of which can be greatly enhanced ~~far more greatly~~ with the addition of zinc ion to a culture medium therefor. The invention further provides a process for producing D-aminoacylase using the transformed microorganism.

The transformed microorganism of the invention is a microorganism having acquired high-expression ability to produce D-aminoacylase in a zinc ion-containing culture medium~~,~~. This transformed organism may be prepared by inserting a D-aminoacylase-producing gene into a zinc tolerant host microorganism ~~with zinc tolerance a D-aminoacylase producing gene~~ wherein the expression of a gene product ~~of which~~ of the

inserted gene is enhanced in the presence of zinc ion. The transformed microorganism is a microorganism transformed with a D-aminoacylase-producing gene, and due to the addition of zinc ion to the culture medium, the D-aminoacylase-producing potency thereof can be enhanced to maximum.

In the transformed microorganism of the invention, the D-aminoacylase-producing gene more preferably has a nucleotide sequence of SEQ ID NO: 1 ~~in the sequence listing~~ or a nucleotide sequence hybridizing to the nucleotide sequence of SEQ ID NO: 1 ~~in the sequence listing~~ under stringent conditions and effectively encoding D-aminoacylase. It has been confirmed that a D-aminoacylase-producing gene having a nucleotide sequence of SEQ ID NO: 1 ~~in the sequence listing~~ is a gene the expression of a gene product of which can greatly be enhanced in the presence of zinc ion. Further, a gene of a nucleotide sequence hybridizing to the nucleotide sequence of SEQ ID NO: 1 ~~in the sequence listing~~ under stringent conditions and effectively encoding D-aminoacylase can be expected to have similar characteristics.

More preferably, in the transformed microorganism of the invention, the ~~a~~ host microorganism is Escherichia coli. It has been confirmed that Escherichia coli has zinc tolerance. Further, the mycological and physiological properties, culture conditions and maintenance conditions of Escherichia coli are well known. Thus, the production of D-aminoacylase at high

efficiency can be done under readily controllable conditions.

Still more preferably, in the transformed microorganism of the invention, a D-aminoacylase-producing gene which is to be inserted into a host microorganism is subjected to at least one of the following modifications (1) and/or (2). (1) A ~~M~~modification for improving the translation efficiency, comprising designing a specific nucleotide sequence (GAAGGA) (SEQ ID NO: 3) in the ribosome-binding site and inserting the nucleotide sequence in the position of the ninth base upstream of the translation initiation point of the gene. This modification improves the translation efficiency of the D-aminoacylase-producing gene. (2) A ~~M~~modification for improving the gene expression efficiency, comprising creating a HindIII recognition site of Escherichia coli in the upstream and downstream of the gene, subsequently purifying and excising the resulting gene, and ligating the gene into an expression vector. This modification improves the expression efficiency of the D-aminoacylase-producing gene.

A zinc-tolerant microorganism is used as a host microorganism for obtaining a transformed microorganism in accordance with the invention. More specifically, a microorganism should be used, the growth potency of which in culture media, as measured on the basis of increase or decrease of the cell weight (A660 nm), is not ~~so much~~ significantly inhibited by the addition of zinc ion. ~~One of the standards~~

~~to evaluate zinc tolerance is as follows.~~ Zinc tolerance may be evaluated by comparing the cell weight of microorganisms grown in a zinc-free culture medium with the cell weight of the same microorganism grown in a medium containing zinc. On the basis of the cell weight (A660 nm) of the microorganism in a zinc-free culture medium, the cell weight in the same culture medium under the same conditions except for the addition of 2 mM zinc either increases, or decreases within a range of 10 %. Otherwise, the above-mentioned cell weight in the same culture medium under the same conditions except for the addition of 5 mM zinc increases, or decreases within a range of 20 %.

Although the taxonomical group of the host microorganism is not limited, it is generally preferable to use such host microorganisms that have well known ~~the~~ morphological and physiological properties ~~are well known and~~ and for which the culture conditions and maintenance conditions are also well known. A preferable example of such a host microorganism is Escherichia coli. Compared with Escherichia coli, microorganisms of the species Alcaligenes xylosoxidans including A-6 strain do not have zinc tolerance.

The means for inserting a D-aminoacylase-producing gene into a host microorganism is not specifically limited. For example, a D-aminoacylase-producing gene may be inserted into either a plasmid or a bacteriophage by ligation to plasmid or bacteriophage DNA ~~an insertion method comprising plasmid~~

~~ligation, an insertion method comprising ligation to bacteriophage DNA, and the like may be arbitrarily selected as required~~.

The D-aminoacylase-producing gene in accordance with the invention is a gene selectively producing D-aminoacylase ~~alone between~~ as opposed to producing both D-aminoacylase and L-aminoacylase~~,~~. ~~and~~ This gene is of a type in which the activity expression is enhanced in the presence of zinc ion in the culture medium. As a preferable example of such D-aminoacylase-producing gene, the gene with the nucleotide sequence of SEQ ID NO: 1 ~~in the sequence listing~~ has been confirmed. Further, genes of nucleotide sequences hybridizing to the nucleotide sequence of SEQ ID NO: 1 ~~in the sequence listing~~ under stringent conditions and effectively encoding D-aminoacylase are also preferable, except for genes which do not actually enhance the activity expression with zinc ion in the culture medium.

The D-aminoacylase-producing gene with the nucleotide sequence of SEQ ID NO: 1 was obtained from the Alcaligenes xylosoxidans subsp. xylosoxidans A-6 strain. The A-6 strain is a D-aminoacylase-producing strain obtained from soil in nature via screening.

The process for producing D-aminoacylase in accordance with the invention comprises culturing any transformed microorganism as described above in a culture medium containing

zinc ion, and obtaining D-aminoacylase from the culture. Zinc ion can be provided by adding an appropriate amount of a zinc compounds~~s~~, such as zinc chloride and zinc sulfate, to the culture medium. This process enables ~~to produce~~ the production of D-aminoacylase at a high efficiency.

In the process for producing D-aminoacylase in accordance with the invention, the concentration of zinc ion contained in the culture medium is preferably controlled to be in the range of 0.1 to 10 mM. This process enables to optimize the zinc ion concentration in the culture medium, and to produce D-aminoacylase at a particularly high efficiency.

In the process for producing D-aminoacylase, other procedures and conditions for carrying out the process are not specifically limited. Nevertheless, the culture is preferably carried out in a nutritious culture medium containing tac promoter-inducing substances (for example, isopropyl thiogalactoside (IPTG), lactose and the like) as inducers. Further, the concentration of lactose then is preferably adjusted to about 0.1 to 1 %.

~~Brief Description of the Drawings~~

~~Fig. 1 schematically depicts the plasmid used for ligating with the D-aminoacylase producing gene. Fig. 2 schematically depicts the plasmid ligated with the D-aminoacylase producing gene.~~

Best Mode for Carrying out the Invention

The best mode ~~Best modes~~ for carrying out the invention ~~are~~ is described below ~~together~~ in conjunction with a comparative example. The invention is ~~never~~ not limited to the best mode ~~these modes~~ for carrying out the invention.

~~(Obtainment of gene and determination of nucleotide sequence)~~ Obtaining the D-aminoacylase gene and determining its nucleotide sequence.

The chromosomal DNA obtained from Alcaligenes xylosoxidans subsp. xylosoxidans A-6 strain was partially digested with restriction endonuclease Sau3AI, to obtain by fractionation DNA fragments of 2 to 9 Kb. The resulting DNA fragments were inserted in and ligated ~~at~~ into the BamHI recognition site of a known plasmid, pUC118. Escherichia coli JM109 was transformed with the ligated plasmid[,] to obtain an ampicillin-resistant transformant strain. Among the thus obtained transformant strains, a strain with [a potency of] the ability to selectively ~~producing~~ produce D-aminoacylase alone was obtained. The transformant strain with ~~the potency retained the~~ this ability contained a plasmid with a 5.8-Kb insert fragment.

The 5.8-Kb insert fragment in the plasmid was trimmed down to deduce the position of the D-aminoacylase-producing gene. According to general methods, then, the nucleotide sequence as shown in SEQ ID NO:1 ~~in the sequence listing~~ was

determined for the DNA of about 2.0 Kb. An amino acid sequence corresponding to the nucleotide sequence is also shown in the sequence listing. Consequently, an open reading frame (ORF) consisting of 1452 nucleotides starting from ATG was confirmed.

~~(Gene modification)~~

Modification of the D-aminoacylase gene

From the plasmid with the 5.8-Kb insert fragment was excised a 4-Kb DNA fragment via BamHI-HindIII digestion, which was then ligated into a known plasmid pUC118 to construct a ligated plasmid pAND118. Using the resulting plasmid, site-directed mutagenesis using primers was effected, to thereby prepare a ribosome-binding site (RBS)-modified plasmid pANSD1.

Using the plasmid pANSD1 as template, site-directed mutagenesis using primers was effected, thereby to prepare a plasmid pANSD1HE having an EcoRI recognition site and a HindIII recognition site immediately upstream the RBS and immediately downstream the ORF, respectively.

Then, the plasmid pANSD1HE was digested with restriction endonucleases EcoRI and HindIII to prepare a 1.8-Kb DNA fragment, which was inserted in and ligated at the EcoRI-HindIII site in the plasmid pKK223-3 shown in Fig. 1 to obtain the plasmid pKNSD2 shown in Fig. 2.

~~(Transformed Escherichia coli)~~

Transformation of Escherichia coli with the D-aminoacylase gene

The plasmid DNA was inserted into a host strain derived from the Escherichia coli K-12 strain by the D. HANAHAN's method (DNA Cloning, Vol.1, 109-136, 1985), thereby to obtain a transformed Escherichia coli (E. coli) TG1/pKNSD2.

~~(Zinc tolerance of bacterial strain as gene source)~~

Zinc-tolerance of the bacterial strain from which D-aminoacylase gene was obtained

The Alcaligenes xylosoxidans subsp. xylosoxidans A-6 strain was cultured at 30°C for 24 hours in a culture medium (pH 7.2, zinc-free) containing 0.2 % potassium dihydrogen phosphate, 0.2 % dipotassium hydrogenphosphate, 2 % polypeptone, 0.02 % magnesium sulfate and 1 % glycerin, and in culture media of the same composition but with addition of zinc oxide to concentrations 0.2 mM, 2.0 mM and 5.0 mM, respectively. After culturing, the cell weight (A660 nm) was measured to evaluate the zinc tolerance. Then, the pH of the culture media after culturing was measured. The results are shown in the column of "A-6 bacteria" in Table 1.

Table 1

Microbial strain	Zinc concentration (mM)	Post-culture pH	Cell weight (A660)	Relative value (%)
A-6 bacteria	0.0	7.58	8.09	100.0
	0.2	7.62	7.75	95.8
	2.0	7.56	5.23	64.6
	5.0	7.68	3.34	41.3
TG1 (host bacterium)	0.0	5.01	5.68	100.0
	0.2	4.99	5.93	104.4

	2.0	4.98	5.55	97.7
	5.0	5.01	4.98	87.7
pKNSD2/TG1 (recombinant bacterium)	0.0	5.00	6.45	100.0
	0.2	5.01	6.70	103.9
	2.0	4.98	6.09	94.4
	5.0	5.01	5.47	84.8

Table 1 shows that the cell weight of the A-6 strain in the zinc-added culture media was greatly decreased (decreased by about 35 % in the 2.0 mM zinc-added culture medium and by about 60 % in the 5.0 mM zinc-added culture medium), compared with the cell weight of the A-6 strain in the zinc-free culture medium. This indicates that the A-6 strain was not zinc-tolerant.

~~(Zinc tolerance of host bacterium)~~

<u>Zinc tolerance of host bacterium</u>

The zinc tolerance of the strain derived from the <u>Escherichia coli</u> K-12 strain used as the host bacterium was examined, using a culture medium of the same composition as for the A-6 strain, by measuring the cell weight (A660 nm) in the same manner. The results are shown in the column of "TG1 (host bacterium)".

Table 1 shows that the cell weight of the host bacterium in the zinc-added culture media was not so greatly decreased (decreased by about 3 % in the 2.0 mM zinc-added culture medium and by about 12 % in the 5.0 mM zinc-added culture medium, and even increased in the 0.2 mM zinc-added culture medium), compared

with the cell weight of the host bacterium in the zinc-free culture medium. This indicates that the host bacterium was zinc-tolerant.

~~(Zinc tolerance of transformed Escherichia coli)~~

Zinc tolerance of transformed *Escherichia coli*

The zinc tolerance of the transformed *Escherichia coli* *(E.coli)* TG1/pKNSD2 was examined using a culture medium of the same composition as for the A-6 strain by measuring the cell weight (A660 nm) in the same manner. The results are shown in the column of "pKNSD2/TG1 (recombinant bacterium)".

Table 1 shows that the cell weight of the transformed bacterium in the zinc-added culture media was not so greatly depressed (decreased by about 5 % in the 2.0 mM zinc-added culture medium and by about 15 % in the 5.0 mM zinc-added culture medium, and even increased in the 0.2 mM zinc-added culture medium), compared with the cell weight of the transformed bacterium in the zinc-free culture medium. This indicates that the transformed Escherichia coli was zinc-tolerant.

(Effect of zinc addition on transformed Escherichia coli)

The transformed Escherichia coli (E. coli) TG1/pKNSD2 was pre-cultured in a culture medium (pH 7.0) containing 1 % bacto-tryptone, 0.5 % bacto-yeast extract, 0.5 % sodium chloride and 100 μg/ml ampicillin, at 30°C for 16 hours.

Subsequently, the post-preculture transformed Escherichia coli was cultured at 30°C for 24 hours in a culture

medium (pH 7.0, zinc-free) containing 0.2 % potassium dihydrogen phosphate, 0.2 % dipotassium hydrogen phosphate, 2 % polypeptone, 0.01 % magnesium sulfate, 1 % glycerin and 0.1 % lactose as an inducer, and culture media of the same composition but with addition of zinc oxide to concentrations 0.2 mM and 2.0 mM. Additionally, the broth-out pH of the culture broth as well as the enzyme activity (U/mL) of D-aminoacylase in the culture broth (A660 nm) was measured.

Consequently, the enzyme activity in the 0.2 mM zinc-added culture medium was 58.85 U/mL (broth-out pH of 5.03) and the enzyme activity in the 2.0 mM zinc-added culture medium was 109.79 U/mL (broth-out pH of 5.11), compared with the enzyme activity of 21.78 U/mL in the zinc-free culture medium (broth-out pH of 5.05). Thus, it has been confirmed that the addition of zinc ion, at least within a predetermined concentration range greatly improves the D-aminoacylase-producing potency.

For comparison, additionally, the A-6 strain was pre-cultured in the culture medium for preculture (no ampicillin was however added) under the same conditions, and was then cultured in the culture medium of the same composition for culture, except for the change of the inducer from 0.1 % of lactose to 0.1 % of N-acetyl-D, L-leucine. Then, the broth-out pH of the culture broth as well as the enzyme activity (U/mL) of D-aminoacylase in the culture broth (A660 nm) was assayed.

Consequently, the enzyme activity in the 0.2 mM zinc-added

culture medium was 0.12 U/mL (broth-out pH of 7.48) and the enzyme activity in the 2.0 mM zinc-added culture medium was 0.29 U/mL (broth-out pH of 7.43), compared with the enzyme activity of 0.29 U/mL in the zinc-free culture medium (broth-out pH of 7.47). Thus, no effect of zinc ion addition on the improvement of the D-aminoacylase-producing potency could be confirmed.

Industrial Applicability

As described above, D-aminoacylase, as an industrially useful enzyme, can be produced highly efficiently and selectively by using the transformed microorganism of the invention.